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**Title:** FLOW CYTOMETRIC METHODS FOR ASSAYING DAMAGE TO RESPIRATORY TRACT CELLS

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FLOW CYTOMETRIC METHODS FOR ASSAYING  
DAMAGE TO RESPIRATORY TRACT CELLS

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ABSTRACT

This paper summarizes results of experiments designed to develop automated flow-analysis assay methods for discerning damage to exfoliated respiratory tract cells in model test animals exposed by inhalation to physical and chemical agents associated with the production of synthetic fuels from oil shale, the specific goal being the determination of atypical changes in exposed lung macrophages and epithelial cells. Animals were exposed to oil shale particulates (raw and spent), silica, and ozone, and respiratory tract cells were obtained by lavaging the lungs with normal saline. Cell samples were stained with fluorescent dyes specific for different biochemical parameters and analyzed as they flowed through a chamber intersecting a laser beam(s) of exciting light where sensors measured fluorescence and light scatter (cell size) on a cell-by-cell basis. Cellular parameters proportional to optical signals were displayed as frequency distribution histograms. Cells also were separated according to cytological features and identified. The basic features of the methodology are presented, along with examples of results that illustrate characterization and analysis of normal and exposed respiratory tract cells based on DNA content, total protein, size, and phagocytic activity.

INTRODUCTION

The application of advanced flow cytometric instrumentation to measure cytological and biochemical properties of respiratory tract cells provides a new approach for assessing potential damage to lung epithelium exposed by inhalation to toxic environmental pollutants associated with the production of synthetic fuels from oil shale and coal.<sup>1-5</sup> This includes development of automated cytological methods for determining atypical changes in exfoliated respiratory tract cells from experimental animals, the end objective being examination of sputum samples from exposed humans. To develop analytical flow-analysis methods for quantitative assessment of cellular damage,

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automated cell-analysis and sorting instrumentation<sup>6-8</sup> is being applied to study respiratory tract cells from hamsters exposed to particulates of oil shale, silica, and ozone. This includes the acquisition of exfoliated lung cells by lavaging the respiratory tract with normal saline; utilization of fluorescence staining methods to measure cellular biochemical parameters; and exposure of experimental animals to physical and chemical toxicants, followed by flow cytometric analysis. Examples of results from initial studies involving measurement of DNA content and total protein in normal and exposed respiratory tract cells are presented, along with a brief description of the instrumentation technique. A new method for quantitating pulmonary macrophage phagocytosis in rats using fluorescent microspheres also is under development. This technology provides a new approach for studying the mechanisms of damage to respiratory tract cells, with future anticipated results serving to assist in estimating risks, evaluating dose-damage relationships, and establishing guidelines for determining exposure levels to humans.

#### MATERIALS AND METHODS

The principle of measurement is illustrated in Fig. 1. Normal and exposed respiratory tract cells composed of macrophages, leukocytes, ciliated columnar and basal undifferentiated cells stained with fluorescent dyes were analyzed in liquid suspension as they flowed through a chamber intersecting a laser beam(s) of exciting light.<sup>9,10</sup> Multiple sensors measured fluorescence and light-scatter optical signals on a cell-by-cell basis. Cellular parameters proportional to optical measurements (e.g., DNA

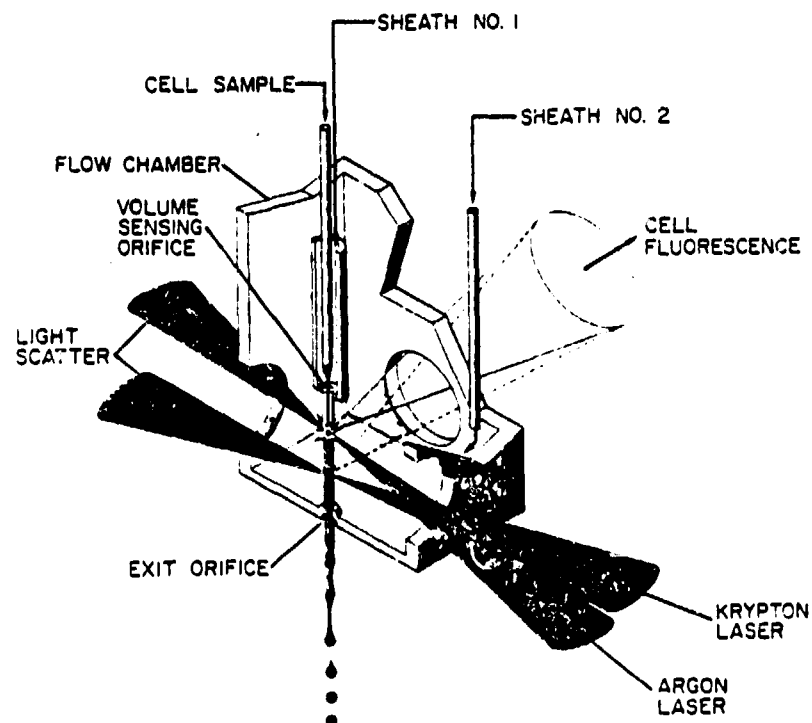


Fig. 1. Cutaway view of the multiparameter cell separator flow chamber, illustrating dual-laser excitation.

content, total protein, cell size, and phagocytic activity) were displayed as frequency distribution histograms using a multichannel pulse-height analyzer. Cells also were separated according to various cytologic parameters and identified microscopically.

To study cellular changes in animals exposed to particulates of oil shale and silica, Syrian hamsters were injected intratracheally with 10 mg of ball-milled (2- to 7- $\mu$ m diameter range) raw and spent oil shale and silica (4- $\mu$ m mean diameter) suspended in 0.2 ml of normal saline. Hamsters were exposed also to 0.2 ml of saline alone. Raw shale (type 2) was obtained from Anvil Points, Colorado. The two spent shales (types 1 and 2) were from solid heat transfer and gas combustion processes, respectively. Silica was obtained from the Pennsylvania Glass and Sand Corporation. Hamsters anesthetized with "Brevital" (5 mg) prior to intratracheal instillation of particulates and saline via the oral cavity were returned to the colony. Animals were sacrificed by pentobarbital injection 28, 35, and 42 days later. The lungs were then lavaged four times with saline to obtain exfoliated cells, which were fixed in 35% ethanol prior to staining for DNA content with mithramycin,<sup>11,12</sup> excited at 457 nm wavelength (argon laser), and analyzed for fluorescence properties.

Syrian hamsters also were exposed to acute levels of ozone (4 ppm for 4 hr) and sacrificed at different times ranging from 0 to 56 hr after termination of exposure. Respiratory tract cells were obtained at sacrifice using pentobarbital, followed by lavaging the lungs with saline, fixing in ethanol, staining with mithramycin (DNA content), and analysis.

Normal hamster respiratory tract cells also were characterized initially based on DNA content and total protein. Cells were obtained by sacrifice, followed by lung lavage using saline. DNA content and total protein were measured by fixing the cells in 35% ethanol, staining with mithramycin (DNA) and rhodamine 640 (protein), and analyzed for two-color fluorescence properties by exciting bound fluorochromes at 457 nm (argon laser) and 468 nm (krypton laser), respectively.<sup>10</sup>

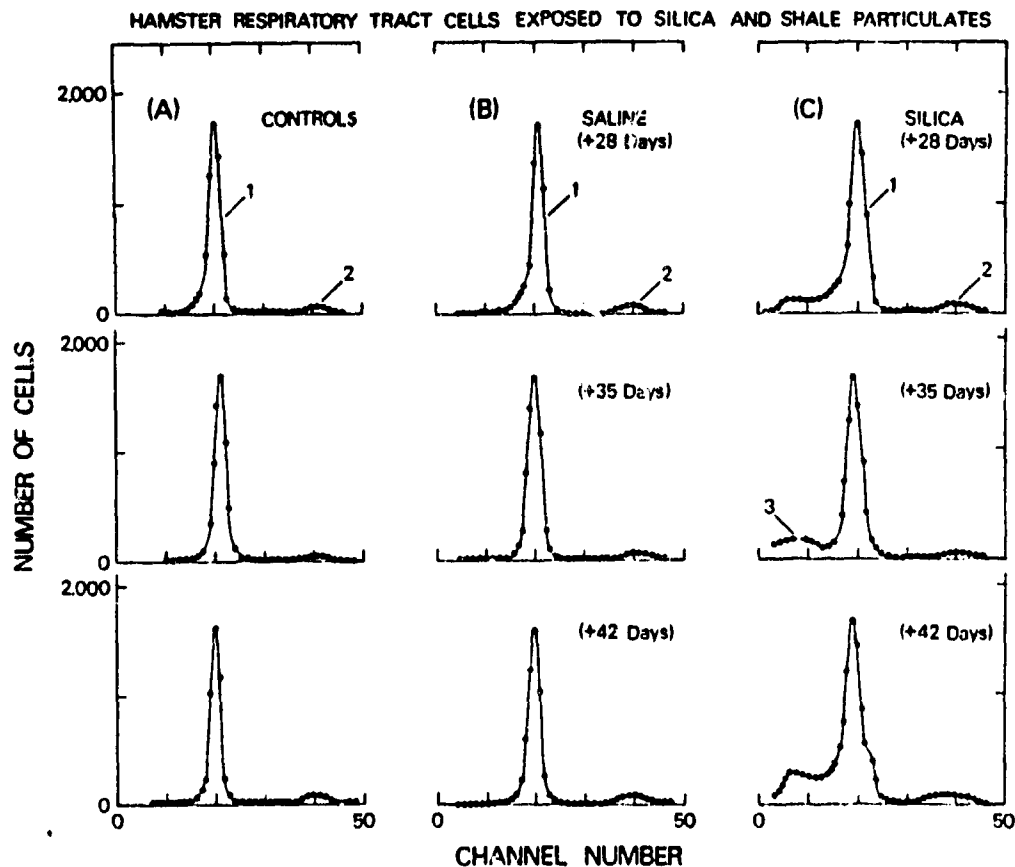
To develop an automated method for quantitating alveolar macrophage phagocytosis, normal Sprague-Dawley rats were anesthetized by inhalation of Metafane. The trachea was then intubated with a blunt needle via the oral cavity, and 1 to 2 x 10<sup>7</sup> polystyrene latex spheres (fluorescent) of 1.83- $\mu$ m diameter suspended in 0.5 ml of saline were delivered to the respiratory tract. After 2 hr, rats were sacrificed by pentobarbital injection and their lungs lavaged with 4 ml of saline (four times). Cells were fixed in 35% ethanol, rinsed and resuspended in saline, excited at 457 nm (argon laser), and analyzed for fluorescence (phagocytized spheres) and light scatter (size).

## RESULTS AND DISCUSSION

### DNA Measurements: Respiratory Tract Cells Exposed to Oil Shale Particulates and Silica

To initiate studies with classes of particulates and known toxic agents, hamsters were exposed to raw and spent oil shale particulates and

silica by intratracheal injection. Since DNA content distributions showed no significant changes compared to controls up to 28 days after exposure, it was decided to examine respiratory tract cells that had been exposed 28 days or more. Figure 2 shows the DNA content per cell distribution of respiratory tract cells from hamsters exposed to saline, silica, and raw and spent oil shale particulates 28, 35, and 42 days after exposure. DNA content distributions of normal control animals are shown in Fig. 2A. Peak 1 represents cells having 2C DNA content and peak 2 binucleated cells and doublets (4C DNA content).<sup>5</sup> DNA content distributions of lung cells from hamsters exposed to saline (Fig. 2B) closely resemble controls. However, DNA content distributions of lung cells from hamsters exposed to silica (Fig. 2C), which appear normal at 28 days postexposure, begin to show atypical changes at 35 and 42 days. A third region has appeared to the left of peak 1, which is most likely dead cells. At 42 days, cells within region 3 have increased and a shoulder is beginning to develop on the right side of peak 1. The percentage of binucleated cells appears to be increasing also. Preliminary DNA content distributions of lung cells exposed to raw and spent oil shale are shown in Figs. 2D, 2E, and 2F. Figure 2D illustrates DNA content distributions of lung cells exposed to type 1 spent shale. These distributions appear nearly normal, with the exception that the left side of peak 1 is skewed.



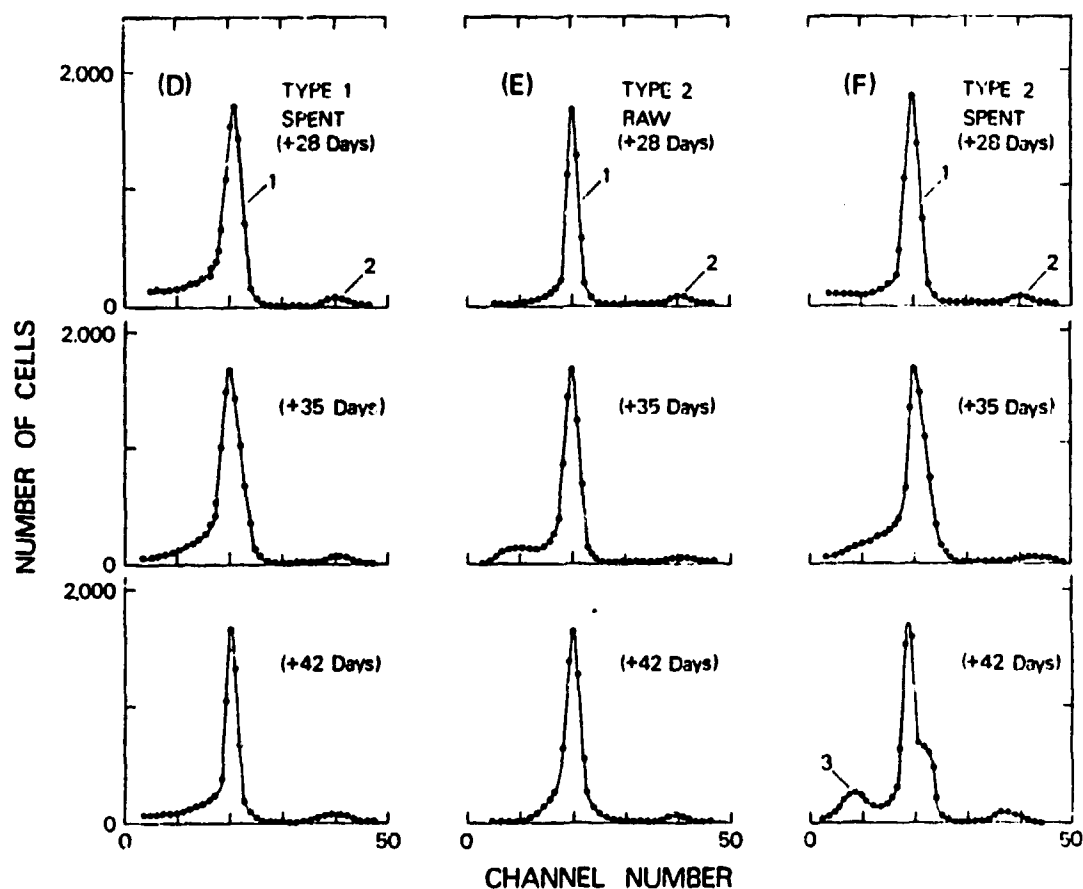


Fig. 2. DNA content frequency distribution histograms of hamster respiratory tract cells exposed (intratracheal injection) to saline, silica, type 1 spent shale, and type 2 raw and spent shale prior to sacrificing 28, 35, and 42 days later. Cell samples were obtained by lung lavage, fixed in 35% ethanol, stained with mithramycin, and analyzed for fluorescence. Types 1 and 2 spent shales were obtained from solid heat transfer and gas combustion processes, respectively.

DNA content distributions from respiratory tract cells exposed to type 2 raw and spent shale are shown in Figs. 2E and 2F, respectively. Distributions from hamsters exposed to raw shale appear nearly normal; however, DNA content distributions from hamsters exposed to spent shale show atypical changes 35 and 42 days postinstillation. A definite shoulder appeared on the right side of peak 1, and the number of cells within region 3 increased. These changes were better observed by increasing the amplifier gain of the fluorescence channel, thus centering peak 1 in channel 30 of the multichannel pulse-height analyzer (Fig. 3). Peak 1 now shows a well-defined region of cells to the right side that is similar to the results from hamsters exposed to ozone, as described below. Although these cells have not been identified at this time, experiments are under way to determine the cell types present in peaks 1 and 3.

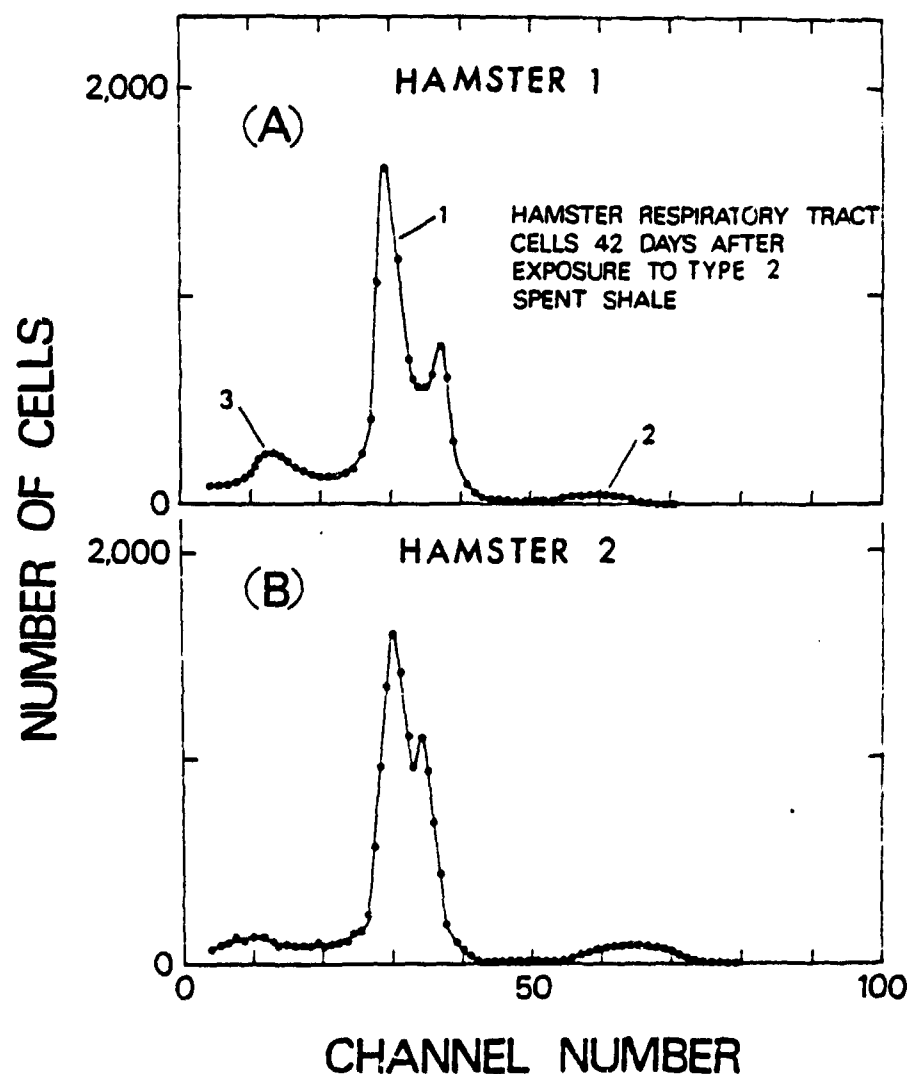


Fig. 3. DNA content frequency distribution histograms of hamster respiratory tract cells exposed (intratracheal injection) to type 2 spent shale prior to sacrificing 42 days later. Cell samples were obtained by lung lavage, fixed in 35% ethanol, stained with mithramycin, and analyzed for fluorescence. Type 2 spent shale was obtained from a gas combustion process.

#### DNA Measurements: Respiratory Tract Cells Exposed to Ozone

Hamsters were exposed to acute levels of ozone (4 ppm for 4 hr) and sacrificed at different times after exposure. These results are shown in Fig. 4. Figure 4A shows a typical DNA content distribution obtained on a normal (control) hamster. DNA content distributions obtained from a hamster immediately at (0 hr) and 1 hr after exposure are shown in Figs. 4B and 4C. Peaks 1 and 2 both show a general broadening, with an increase in the total number of cells contained in peak 2 (binucleated cells and doublets). DNA content distributions measured on hamster lung cells 3, 5, and 7 hr after exposure (Figs. 4D, 4E, and 4F) appear similar. Peak 1 is divided into two

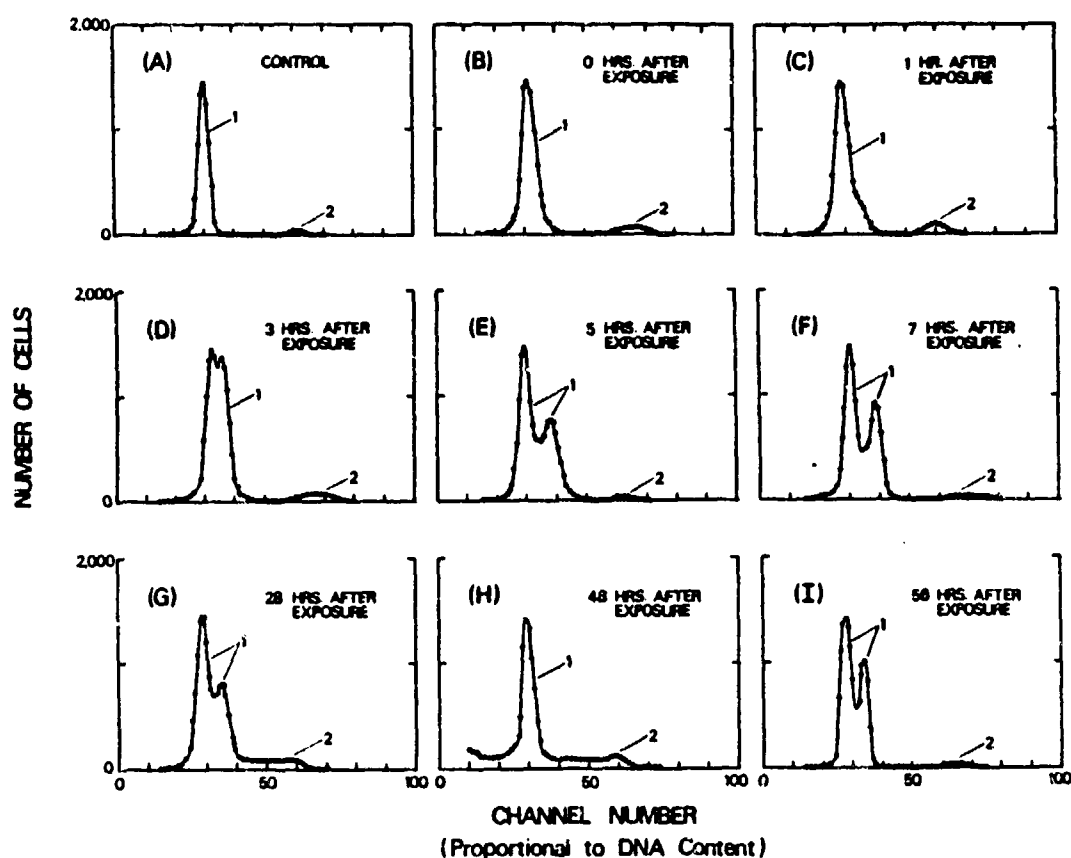


Fig. 4. DNA content frequency distribution histograms of hamster respiratory tract cells fixed in ethanol (70%) and stained with mithramycin. Hamsters were exposed to 4 ppm of ozone for 4 hr prior to obtaining samples at increments ranging from 0 to 56 hr after exposure.

separate parts (bimodal distribution), whereas the number of cells contained within peak 2 has diminished. The DNA distribution of hamster respiratory tract cells 28 hr after exposure is shown in Fig. 4G. This distribution, which is similar to those recorded in Figs. 4D, 4E, and 4F, has an increased percentage of cells between peaks 1 and 2. Figure 4H shows a DNA content distribution for hamster cells 48 hr after exposure, which "resembles" a typical DNA distribution for randomly growing CHO cells in which peak 1 represents  $G_1$ -phase cells (2C DNA content) and peak 2  $G_2$  and M-phase cells (4C DNA content). Cells located between peaks 1 and 2 would then be S-phase cells. In Fig. 4I (56 hr after exposure), the DNA content distribution per cell has reverted back to resemble distributions recorded at earlier times after exposure (Figs. 4D, 4E, and 4F).

These initial results vividly demonstrate the importance of using flow cytometric analysis methods as a new methodology to study the effects of exposure and recovery to known toxic agents. Future experiments will consist of verifying these results, studying other time increments after exposure, and correlating cytology (morphological features, differential

cell counts, etc.) with DNA content measurements. Cells also will be sorted and microscopically identified. DNA measurements also can be used to study cell-cycle kinetics and would thus permit recovery mechanisms to be analyzed dynamically, especially when coupled with other cellular parameters (e.g., protein, enzymes, etc.) using multiparameter analysis methods.

#### DNA-Protein Measurements: Normal Respiratory Tract Cells

A new dual-laser multiparameter flow system has broad potential application in basic cell biology research, including the analysis of respiratory tract cells. This system has been used recently to measure DNA content with dyes having ultraviolet absorption ranges<sup>4</sup> and to analyze DNA content and protein in cells stained with mithramycin and rhodamine.<sup>10</sup> Mithramycin and rhodamine have violet and yellow excitation ranges, respectively, with overlapping emission spectra.<sup>10</sup> This measurement is made possible only through the use of dual-laser excitation. For example, hamster respiratory tract cells have been analyzed recently using this procedure, as illustrated in Fig. 5. Peak 1 of the DNA content distribution represents mononucleated cells (macrophages, leukocytes, etc.) having 2C DNA content and peak 2 binucleated cells and doublets (4C DNA content).

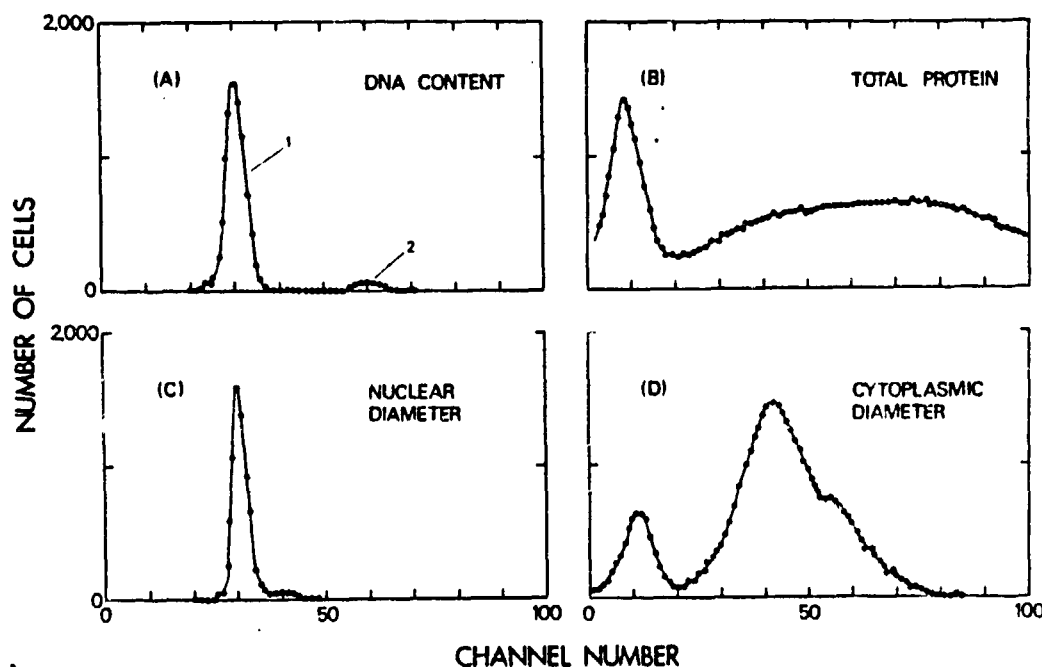


Fig. 5. Frequency distribution histograms of normal hamster respiratory tract cells fixed in ethanol (70%) and stained with mithramycin (DNA content) and rhodamine 640 (total protein): (A) DNA content; (B) total protein; (C) nuclear diameter; and (D) cytoplasmic diameter. The nuclear and cytoplasmic diameter distributions were obtained by measuring the time durations of the fluorescence signals from the nucleus (mithramycin) and cytoplasm (rhodamine), respectively.

Figure 5B, which represents the distribution of protein within the lung cell population, is broad and similar to that previously reported using the propidium iodide-fluorescein isothiocyanate method.<sup>3</sup> The nuclear and cytoplasmic diameter distributions are shown in Figs. 5C and 5D, respectively. Peaks 1 and 2 (cytoplasmic diameter distribution) have been identified recently as being composed of (a) leukocytes and (b) macrophages and epithelial cells, respectively. This new staining and analysis method has broad application in measuring the biochemical and cytological properties of respiratory tract epithelium.

#### Quantitation of Pulmonary Macrophage Phagocytosis

Phagocytic activity, which is the primary function of pulmonary macrophages, is normally measured by exposing test animals to toxic agents, followed by intratracheal injection of micron-sized polystyrene latex particles or bacteria for a fixed time period and lung lavage to remove macrophages, and microscopic enumeration of macrophages containing 1, 2, 3, etc., particles per cell. Described below is a new method to study the mechanisms of phagocytosis of alveolar macrophages from experimental animals exposed to toxicants using 1.83- $\mu$ m diameter polystyrene latex (fluorescent) spheres. Figure 6A shows the fluorescence distribution of phagocytized and nonphagocytized spheres obtained from lavaging the respiratory tract. This distribution was obtained by recording the fluorescence signals from macrophage-ingested spheres and nonphagocytized particles. Peaks 1, 2, and 3 of Fig. 6A represent single macrophages that contain one sphere or a single sphere alone; single macrophages containing two spheres or two spheres stuck together (doublet); and single macrophages containing three spheres or three spheres stuck together (triplet), respectively. To distinguish between macrophages that have phagocytized spheres and nonphagocytized particles, the light-scatter method<sup>6,9</sup> for cell-size determination was used. Since 1.83- $\mu$ m diameter spheres are much smaller than pulmonary cells, they did not appear in the cell-size distribution (Fig. 6B). Peak 1 is thought to be leukocytes and cellular debris. Peak 2 has been identified to represent macrophages that do and do not contain phagocytized spheres. Therefore, by requiring fluorescence signals to be or not to be in coincidence with light-scatter signals (cells), nonphagocytized spheres and macrophages that have phagocytized spheres can be distinguished. For example, Fig. 6C shows the fluorescence distribution of only macrophages that have ingested spheres as obtained by displaying only those fluorescence signals that also scatter light. Cells contained within peaks 1 to 5 (Fig. 6C) represent macrophages having phagocytized 1 to 5, respectively, as identified by sorting cells from each peak.<sup>5</sup> This technique has potential for permitting rapid and accurate determination of phagocytosis and will be used subsequently to assay for toxicity related to macrophage function.

#### ACKNOWLEDGMENTS

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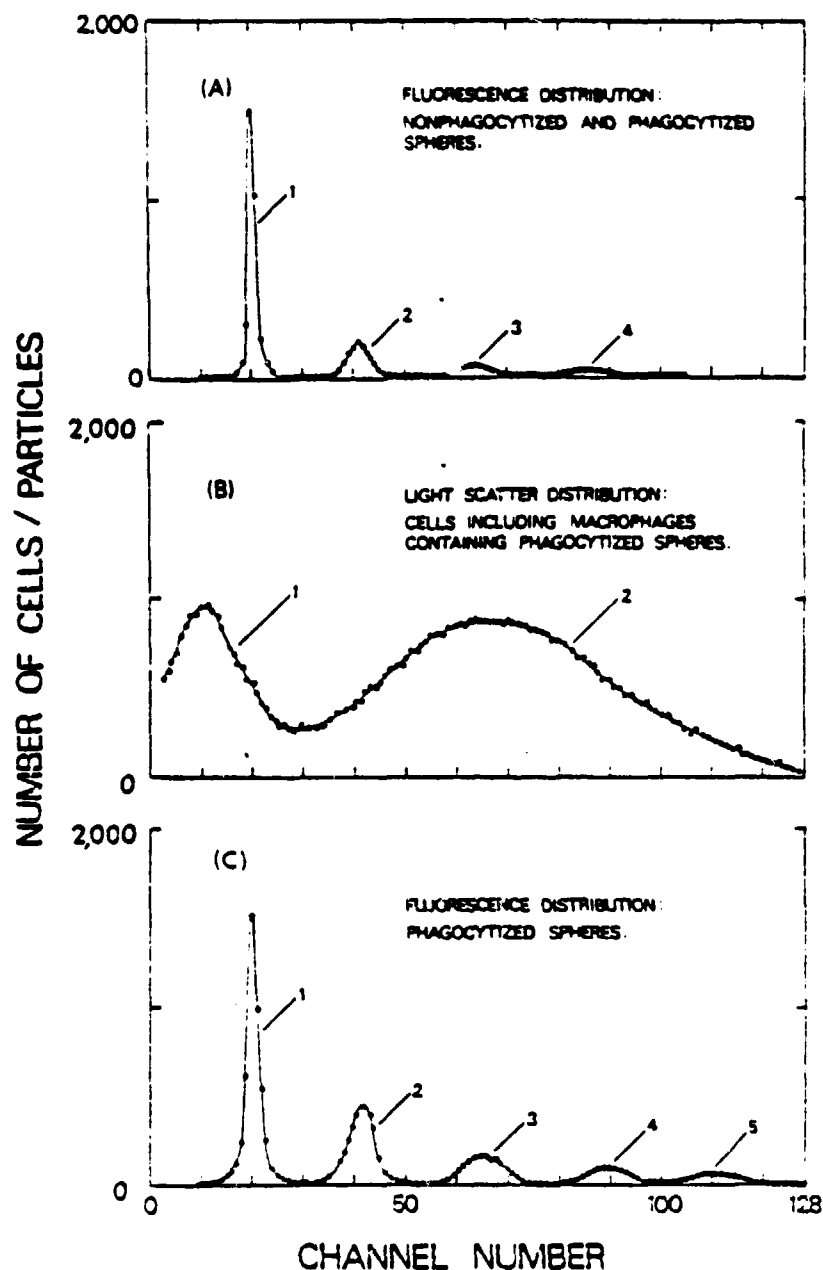


Fig. 6. Frequency distribution histograms of microspheres and cells obtained by sacrificing normal rats and lavaging the lungs with saline 2 hr after instilling  $1$  to  $2 \times 10^7$   $1.83\text{-}\mu\text{m}$  diameter fluorescent spheres in  $0.5$  ml saline: (A) fluorescence distribution of nonphagocytized and phagocytized spheres obtained by recording all fluorescence signals; (B) light-scatter distribution (size) of cells, including macrophages containing phagocytized spheres; and (C) fluorescence distribution of phagocytized spheres obtained by recording only those fluorescence signals associated with light-scatter signals. Cells were fixed in  $3\%$  ethanol prior to fluorescence and light-scatter analysis.

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